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Spatiotemporal Expression of Pregnancy-Specific Glycoprotein Gene *rnCGM1* in Rat Placenta

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ABSTRACT **As a basis towards a better understanding of the role of the pregnancy-specific glycoprotein (PSG) family in the maintenance of pregnancy, detailed investigations are described on the expression of a recently identified rat PSG gene (*rnCGM1*) at the mRNA and protein levels. Using specific oligonucleotide primers, *rnCGM1* transcripts were identified after reverse transcription, polymerase chain reaction, and hybridization with a radiolabelled, internal oligonucleotide. Transcripts were only found in significant amounts in placenta. In situ hybridization visualized *rnCGM1* transcripts at day 14 post coitum (p.c.), in secondary trophoblast giant cells and in the spongiotrophoblast. Only those secondary giant cells lining the maternal decidua were positive. In contrast, primary giant cells did not contain *rnCGM1* mRNA. At day 18 p.c., *rnCGM1* transcripts were almost exclusively detectable in the spongiotrophoblast. No *rnCGM1* transcripts were found in rat embryos of these two developmental stages. Rabbit antisera were generated against the amino-terminal immunoglobulin variable-like domain and against a synthetic peptide containing the last 13 carboxy-terminal amino acids of *rnCGM1*. Both antisera recognized a 124 kDa protein in day 18 rat placental extracts as identified by Western blot analysis. The anti-peptide antiserum recognized a 116 kDa protein in the serum of a 14 day p.c. pregnant rat that is absent from the sera of non-pregnant females. Taken together, these results confirm exclusive expression of *rnCGM1* in the rat trophoblast, but unlike human PSG, negligible or no expression is found in other organs, such as fetal liver or salivary glands, indicating a more specialized function of *rnCGM1*. Its spatiotemporal expression pattern is conducive with a potential role of PSG in protecting the fetus against the maternal immune system and/or in regulating the invasive growth of trophoblast cells.**

Key words: Pregnancy-specific glycoprotein, Carcinoembryonic antigen, Trophoblast giant cell, spongiotrophoblast, embryo

INTRODUCTION

Following fertilization, the developing embryo must overcome a number of major problems to ensure a successful pregnancy. Correct relocation of the embryo to the uterus, followed by controlled invasive growth into the uterine wall, represents one such problem. This intimate contact is necessary for establishing the exchange of nutrients, hormones, waste products, etc. A second major problem faced by the semi-allogenic fetus is the maternal immune system. To avoid rejection, the fetus must escape maternal immunosurveillance, without inducing a state of general immunosuppression. As the trophoblast is the only fetal tissue to encounter maternal cells (Faulk et al., 1982), it is thought to play a major role in regulating these processes. Closer analysis of the trophoblast and its molecular composition may thus help to better understand how fetal/maternal interactions are controlled.

Over the years, a number of placental proteins have been described, one group of which, the pregnancy-specific glycoproteins (PSG), are expressed from early pregnancy onwards in increasing amounts. At term, they represent the major placental protein species secreted into the maternal blood (Lin et al., 1974). These proteins were originally discovered by Tatarinov and Masyukevich (1970) and Bohn (1971) and are heterogeneous with respect to their sizes (Watanabe and Chou, 1988). Using antisera, PSGs have been located to the syncytiotrophoblast (Horne et al. 1976). Although little is known about their function, in vitro inhibition effects on mixed leucocyte cultures and lymphocyte transformation have been reported (Bohn et al., 1976; Cerni et al., 1977; Majumdar et al., 1982), suggesting a possible immunosuppressive function. Other investigations showed that antibodies against PSGs induce abortion of primate or murine pregnancies (Bohn and Weinmann, 1974; Hau et al., 1985), indicating an important role in the maintenance of pregnancy.

Molecular cloning and sequence analysis has identified 11 PSG genes in humans (Khan et al., 1992). Transcripts have so far been identified for 10 of these genes

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(Thompson et al., 1991; Khan et al., 1992). The primary structures of these highly similar PSGs, as derived from cDNA cloning show them to belong to the carcinoembryonic antigen (CEA) family, which in turn can be placed within the immunoglobulin (Ig) superfamily (reviewed in Thompson et al., 1991). All members of the human CEA gene family have a basic backbone of one Ig variable-like (N) domain and a different number of Ig constant-like (A and B) domains. Sequence comparison allows division into two subgroups: the CEA subgroup members encode mainly membrane-bound molecules, whereas the PSG-subgroup encodes secreted proteins (reviewed in Thompson et al., 1991). The expression pattern of human PSG genes has been determined at the mRNA level by Northern analyses, cDNA cloning, and reverse transcriptase-polymerase chain reaction [RT-PCR] (Oikawa et al., 1989; Barnett et al., 1990; Thompson et al., 1990; Streydio and Vassart, 1990). These studies confirm the main site of expression to be the placenta, although PSG transcripts have also been found in salivary glands (Zoubir et al., 1990) and fetal liver (Zimmermann et al., 1989; Khan et al., 1989; Khan and Hammarström, 1990).

For in vivo functional studies of PSGs, animal models must be established. Indeed, PSG-related genes and their transcripts have been identified in the rat (Kodelja et al., 1989; Rebstock et al., 1990; Chen et al., 1992) and more recently in the mouse (Rudert et al., 1992). However, rodent PSGs have a different domain organization and a relatively low sequence conservation compared to their human counterparts. Although they too are secreted, they consist of multiple Ig variable-like (N) domains, ranging from three (Cea-2, Cea-6, rnCGM6) to five (*rnCGM1*) in number, followed by a single Ig constant-like (A) domain. Assignment to the PSG subgroup has only been possible by comparing expression patterns. For this reason, rat PSGs were originally named *Rattus norvegicus* CEA gene-family members (rnCGM).

As a basis for functional studies of the PSGs we have characterized the rat PSG *rnCGM1* as well as the expression pattern of the corresponding gene during placental development and in adult animals.

RESULTS

Analysis of the *rnCGM1* Expression Pattern

Polymerase chain reaction analyses. Expression of *rnCGM1* at the mRNA level was determined by RT-PCR in a number of tissues, including those which have been reported in humans to express PSG. The sequences of the oligonucleotides used as 5′- and 3′-primer were deduced from the L′3 and the N5 domain, respectively (Rebstock et al., 1990). The former region shows very little similarity to other truncated leader regions within *rnCGM1* mRNA or to other, so far identified, PSG mRNAs of rat or mouse. Although *rnCGM1* cDNA contains five related L/N domains (Rebstock et al., 1990) only the expected 845 bp amplification product was obtained with full-length *rnCGM1* cDNA (data not shown). This result and the fact that at least 5 mismatches within the L′3 oligonucleotide are found when compared to the other known members of rat PSG subgroup (Kodelja et al., 1989; Chen et al., 1992) suggest that with this pair of oligonucleotides, only *rnCGM1* mRNA will yield the product of the expected size. With this assay, *rnCGM1* transcripts could be detected in placentae at days 18 and 20 of gestation (Fig. 1a, lanes 2 and 3). No signals were seen with RNAs from fetal liver, adult colon, spleen, kidney, ovary, pancreas, salivary gland, uterus, and testis. The specificity of the assay was shown by hybridization of the PCR products under stringent conditions with an internal oligonucleotide. Strong hybridization was observed with the DNA fragments obtained with placental RNA, whereas marginal signals were seen with RNA from fetal liver, adult colon, pancreas, and testis (Fig. 1b). The intactness of the RNAs was analysed using β-actin primer pairs (Fig. 1c). Since a number of processed β-actin pseudogenes exist (Ng et al., 1985), the same reaction was performed without reverse transcription prior to the PCR, to test for the presence of contaminating genomic DNA. This analysis indicates that a few RNA samples contain DNA since a DNA fragment of the same size was observed with and without reverse transcription (Fig. 1d, lanes 1, 2, 8, and 11). In order to demonstrate that these RNAs are not degraded, a cDNA fragment of the ubiquitously expressed *c-abl* proto-oncogene (Ramakrishnan and Rosenberg, 1989) was amplified (Fig. 1e).

Analysis of placental expression by in situ hybridization. In order to analyze the expression of *rnCGM1* during development and to identify the *rnCGM1*-transcribing cell population in rat placenta, in situ hybridization with [^{35}S]labelled antisense RNA was performed. A plasmid containing the 1,171 bp *SstI*/EcoRI cDNA fragment of λ*rnCGM1*b, which covers the A domain coding region and the 3′-untranslated region, was used for probe synthesis (Rebstock et al., 1990). Under stringent conditions this probe detects two mRNA species of 3.2 and 3.9 kb in rat placenta RNA which could be derived from the same primary *rnCGM1* transcript by alternative polyadenylation. This is suggested by the presence of a poly(A)-addition signal about 200 bp upstream of the 3′-end of λ*rnCGM1*b (Rebstock et al., 1990). However, since other closely related PSG genes exist in rat (Kodelja et al., 1989; Chen et al., 1992) crosshybridization could not be ruled out. Therefore, we used two different oligonucleotides as 5′-primers, one located in the N5 domain, one in the 3′-untranslated region in combination with an oligo(dT)-containing oligonucleotide, which binds at the 5′-end of the poly(A) tail of mRNAs (Leibrock et al., 1989) for PCR analysis of *rnCGM1* mRNA(s). Both reactions yielded two DNA fragments each, which differed by about 700 bp in length. This corresponds to the size difference observed for the two mRNA species. Sequence analysis of the two longer products indeed demonstrated that both mRNAs are

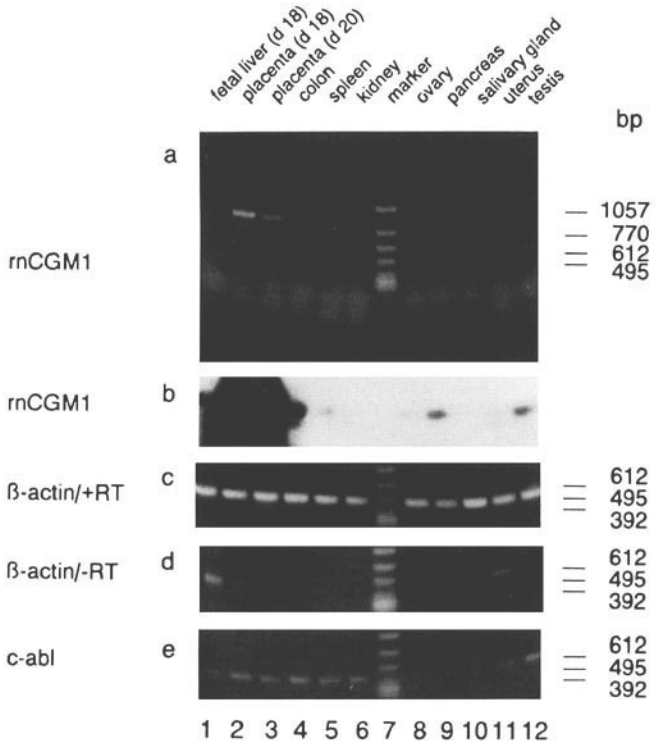

Fig. 1. Analysis of the expression pattern of the rat PSG gene *rnCGM1* by RT/PCR. After reverse transcription, a 845 bp *rnCGM1* cDNA fragment was specifically amplified using oligonucleotides from exon 4 and exon 6 (**a**). Total RNA was from fetal liver (day 18 p.c., **lane 1**); placenta of day 18 and day 20 p.c. (**lanes 2, 3**); colon (**lane 4**), spleen (**lane 5**), kidney (**lane 6**), ovary (**lane 8**), pancreas (**lane 9**), salivary gland (**lane 10**), uterus (**lane 11**) and testis (**lane 12**) from adult animals. In order to assess the specificity of the reaction, the *rnCGM1* cDNA fragments were hybridized to a [^{32}P]labelled oligonucleotide after transfer of the DNA fragments to a nylon membrane (**b**). As a control for the integrity of the RNAs, β-actin (**c**) and c-abl (**e**) cDNA fragments of 525 bp and 406 bp, respectively, were amplified. PCR with the β-actin primers was also performed without reverse transcription prior to amplification to detect contaminating genomic DNA, which yields an identical fragment due to the presence of processed β-actin pseudogenes (**d**). Twenty percent of the amplification products was separated by electrophoresis on 1.8% agarose gels, stained with ethidium bromide and photographed under UV light (a, c–e). *Hin*II-digested bacteriophage ΦX174 DNA was used as size marker (**lane 7**).

derived from the same primary transcript with a second poly(A) signal 659 nucleotide downstream from the first one. The additional sequence of the *rnCGM1* mRNA has been deposited (Genbank accession number L00686). These results indicate that the above mentioned probe is suitable for the specific detection of *rnCGM1* transcripts.

To study the expression of *rnCGM1* in embryonic and extraembryonic tissues, placentae and embryos of day 14 and day 18 of gestation were analysed with this probe. These time points represent well discernible stages of placental development (Beaudoin, 1980). At day 14, the placenta is established. Four layers can be distinguished: the innermost layer comprises the labyrinth zone, where the fetal trophoblast cells are bathed in maternal blood. The fetal blood is separated from the maternal circulation by the embryonic capillary endothelium and a trilaminar trophoblast. The labyrinth is capped by the spongiotrophoblast, a layer of trophoblast cells, which is interspersed with maternal blood lacunae but does not contain fetal blood vessels. The outermost layer is formed by the secondary and primary trophoblast giant cells, respectively. The secondary giant cells are adjacent to the maternal decidua basalis, whereas the primary trophoblast cells line the decidua capsularis. In day 14 placenta, the hybridization signal is confined to the spongiotrophoblast and the secondary giant cell layer (Fig. 2). However, not all cells of these cell layers are positive. Only the secondary giant cells, which are in direct contact with the decidua, express *rnCGM1* mRNA. The staining of this cell population and the distinct border between the positive spongiotrophoblast and the negative labyrinth are evident at higher magnification (Fig. 2C, E). No signals are observed with the sense probe (Fig. 2B,D,F). In contrast, the primary trophoblast giant cells do not express *rnCGM1* mRNA. The rather sharp transition of positive secondary and negative primary giant cells is shown in Figure 3. During further development of the placenta, the relative proportion of the decidua decreases and the giant cell layer is diminished. At day 18, only a few trophoblast giant cells, which are probably metabolically inactive, remain between the spongiotrophoblast and decidua. At this stage of development, the spongiotrophoblast has taken over *rnCGM1* transcription completely (Fig. 4A,C,E). No silver grains are observed over the few remaining giant cells or trophoblast cells of the labyrinth (Fig. 4C,E). In contrast to placenta, no hybridization signals are found in day 14 and day 18 rat embryos (data not shown).

Characterization of the *rnCGM1* Protein

We developed antisera against two different regions of the putative *rnCGM1* protein in rabbits. One region comprised the N1 domain, the amino-terminal domain of the five *rnCGM1* IgV-like domains. This region was expressed in bacteria with histidines fused to its carboxy-terminus, which aided its purification by affinity chromatography on a Ni^{2+}-column. The second antisera was raised against a synthetic peptide, which contains the last 13 carboxy-terminal amino acids and was coupled to keyhole limpet hemocyanin as a carrier. With both antisera a 124 kDa protein could be detected in an extract of day 18 rat placenta after separation on an SDS polyacrylamide gel by Western blot analyses (Fig. 5a, lanes 1 and 3). With the N1 domain antisera, a 75 kDa protein was also strongly stained (Fig. 5a, lane 3). The specificity of the antibody/antigen reactions could be demonstrated either by addition of an excess of coupled peptide (Fig. 5a, compare lanes 1 and 2), which prevented the staining of the 124 kDa antigen, specifically, or by using preimmune serum (data not shown). In contrast, staining by the anti-N1 domain antiserum could not be competed for by addition

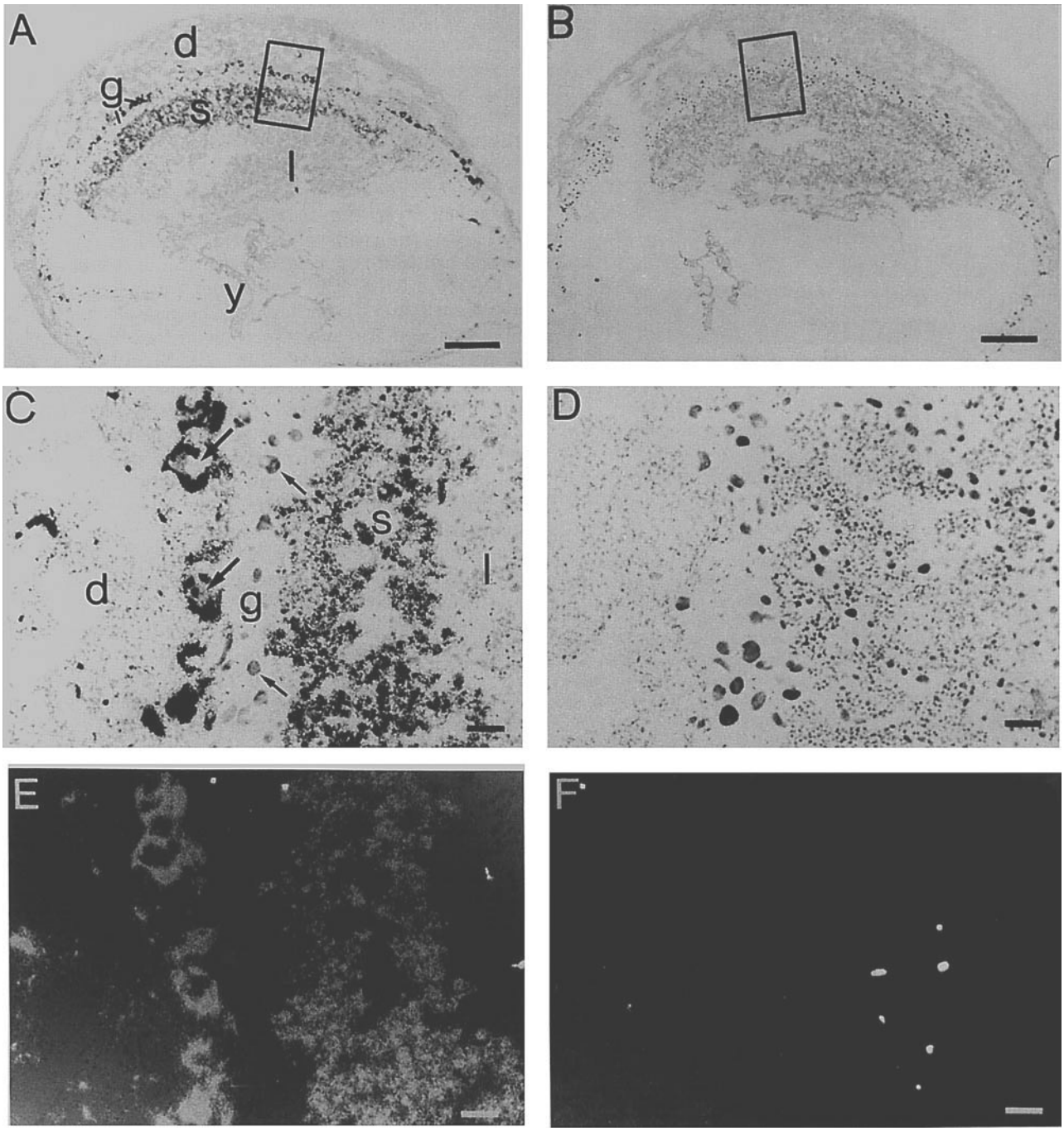

Fig. 2. Localization of *rnCGM1* transcripts in day 14 rat placenta by in situ hybridization. Fixed cryosections of day 14 placentae were hybridized with a *rnCGM1*-specific antisense (**A,C,E**) or as negative control with the sense riboprobe (**B,D,F**), synthesized from the 1,171 bp *Sst*I/*Eco*RI *rnCGM1* cDNA fragment. A and B: Low power view of a section through the maternal and fetal part of the placenta reveals labelling of the fetal trophoblast giant cells (g) and the spongiotrophoblast (s). Maternal decidua (d), fetal labyrinth trophoblast cells (l) and yolk sac (y) are negative. Bars, 1 mm. C and D: Higher magnification views of the boxed regions in A and B showing *rnCGM1* transcripts in giant cells, which are in contact with decidual cells (large arrows), but not in giant cells located toward the spongiotrophoblast (small arrows). Note the complete absence of hybridization signals in the labyrinth. E and F: Dark field views of C and D, respectively. The bright spots represent artefacts. Bars, 0.1 mm.

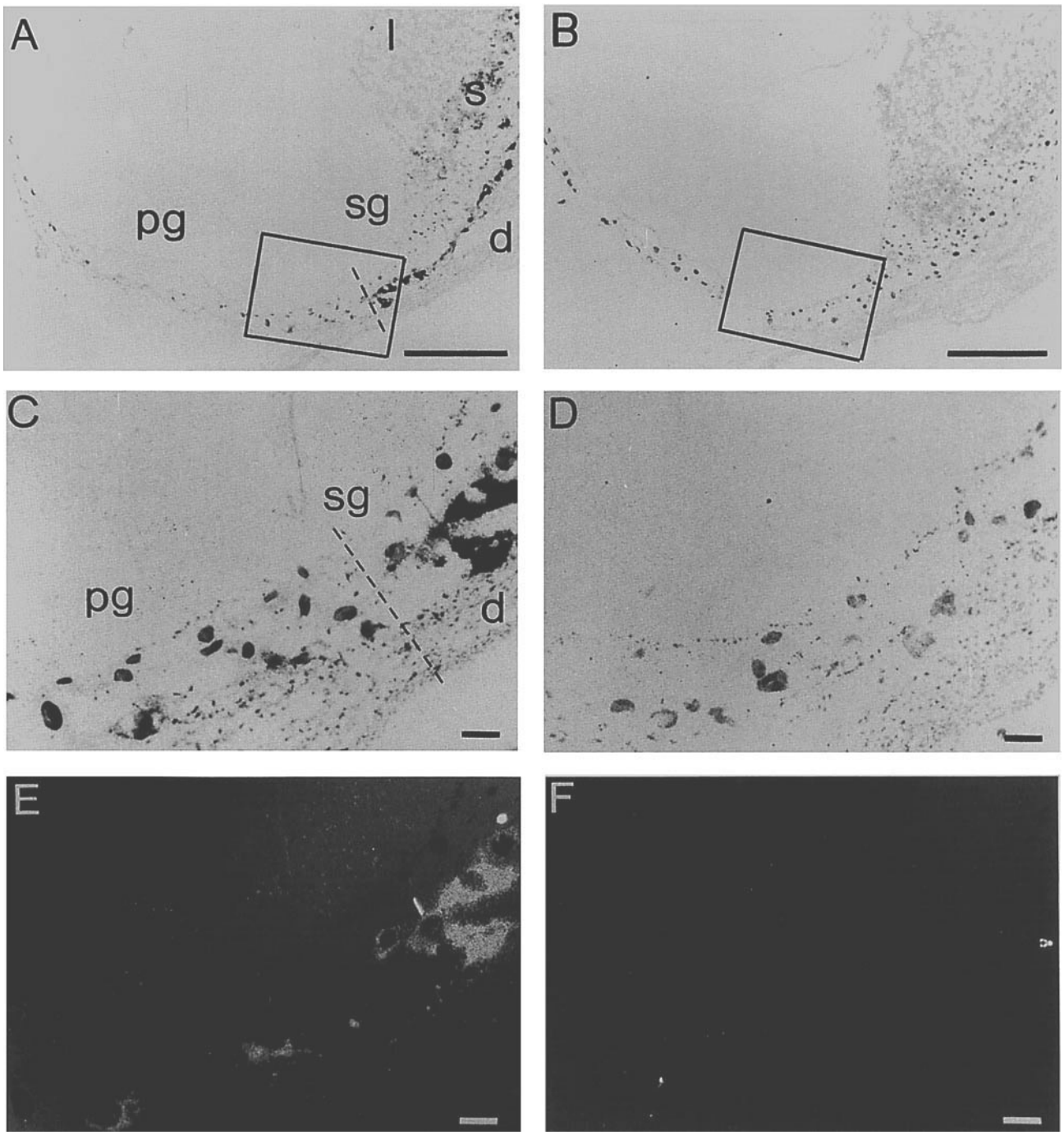

Fig. 3. Secondary, but not primary trophoblast giant cells transcribe *rnCGM1*. **A,B:** Serial section of day 14 placenta to that shown in Figure 2, which highlights the transition of primary to secondary trophoblast giant cells (approximate position indicated by broken line). Bars, 1 mm. **C,D:** Higher magnification views of the boxed regions in A and B showing *rnCGM1* transcripts in secondary (sg), but not in primary trophoblast giant cells (pg). **E,F:** Dark field views of C and D, respectively. Bars, 0.1 mm. s, spongiotrophoblast; d, decidua; l, labyrinth trophoblast cells.

of an excess of the N1 domain fusion protein (Fig. 5a, lanes 3 and 4). This is probably due to the insolubility of the bacterially-expressed N1 domain. A polyclonal antisera raised against human PSG also reacted with a 124 kDa protein (Fig. 5a, lanes 5 and 6). A protein slightly smaller (116 kDa) than the one found in placental extracts could be identified in the serum of a pregnant rat (14 days p.c.) with the anti-peptide antiserum by Western blot analysis (Fig. 5b, compare lanes 1 and 2). This protein was absent in sera of non-

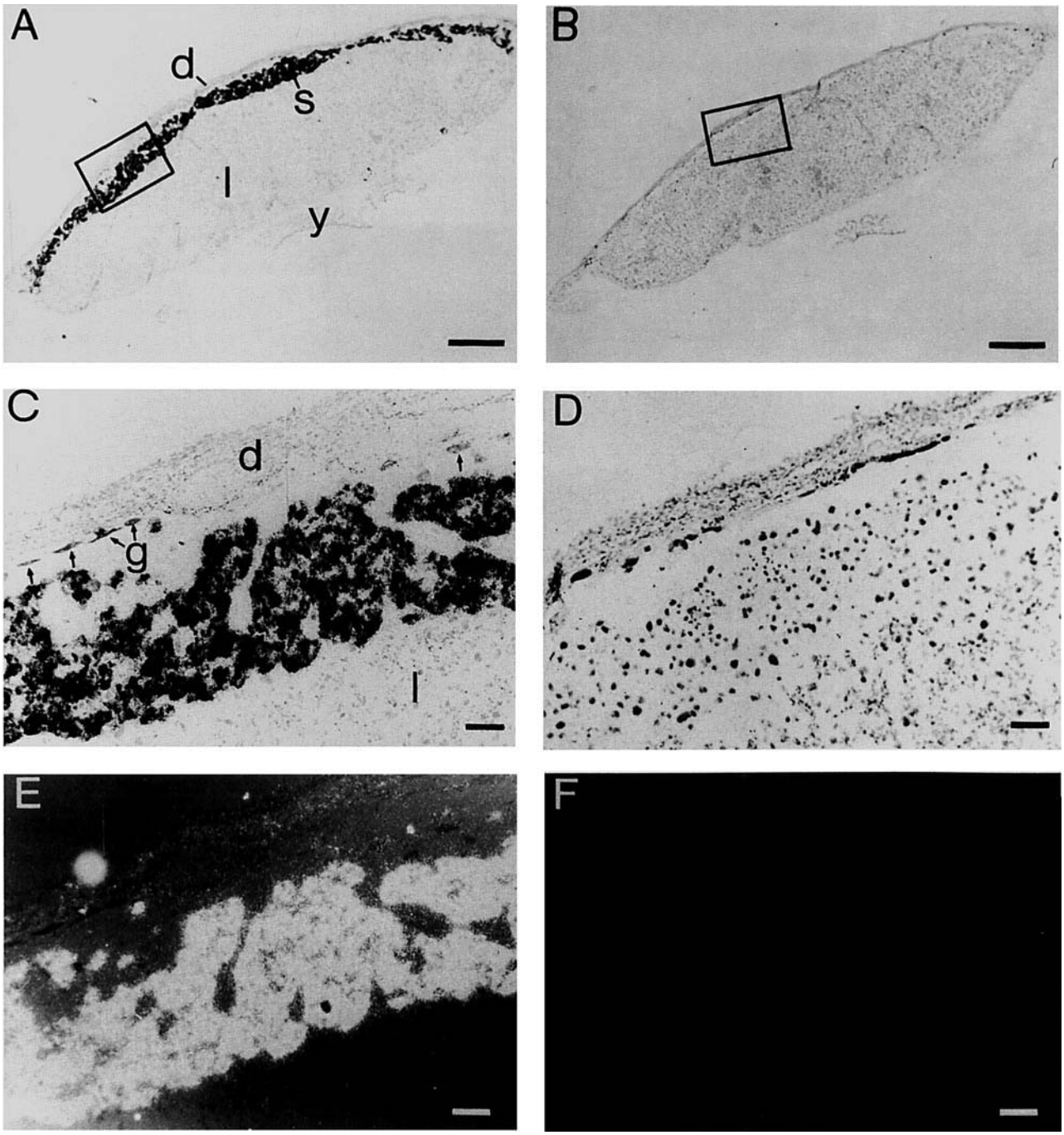

Fig. 4. Localization of *rnCGM1* transcripts in day 18 rat placenta by in situ hybridization. Fixed cryosections of day 18 placentae were hybridized with a *rnCGM1*-specific antisense (**A,C,E**) or as negative control with the sense riboprobe (**B,D,F**), synthesized from the 1171 bp *SstI*/*Eco*RI *rnCGM1* cDNA fragment. A and B: Low power view of a section through the maternal and fetal part of the placenta shows strong labelling of the spongiotrophoblast (s). Maternal decidua (d), fetal labyrinth trophoblast cells (l) and yolk sac (y) are negative. Bars, 1 mm. C and D: Higher magnification views of the boxed regions in A and B demonstrate that the giant cells (arrows) remaining at that stage of placental development are devoid of *rnCGM1* transcripts. E and F: Dark field views of C and D, respectively. Bars, 0.1 mm. d, decidua; g, trophoblast giant cell; s, spongiotrophoblast; l, labyrinth; y, yolk sac.

pregnant females (Fig. 5b, lane 3). Neither *rnCGM1* antisera recognizes native *rnCGM1* protein, since they did not immunoprecipitate rnCGM1 from supernatants of *rnCGM1* transfectants and, in contrast to anti-human PSG antiserum, did not immunostain placental cryosections (data not shown).

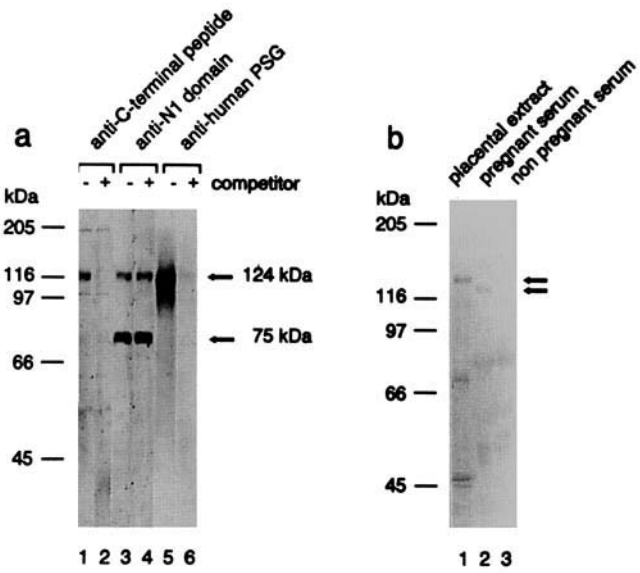

Fig. 5. Western blot analysis of *rnCGM1* protein. Proteins were separated by electrophoresis on 7.5% SDS/polyacrylamide gels, transferred to nylon membranes and incubated with polyclonal antisera or antibodies (dilution 1:300). **a:** Protein extracts of day-18 rat placentae (60 μg) were analysed with anti-C-terminal peptide antiserum (**lanes 1,2**), anti-N1 domain antiserum (**lanes 3,4**) and anti-human PSG antibodies (DAKO) (**lanes 5,6**) in the absence (odd numbers) or presence of 150 μg of peptide conjugate (**lane 2**), 2 mg of recombinant N1 domain protein (**lane 4**) or 50 μg of human PSG (**lane 6**). (**b**) Sera (100 μg of protein) from pregnant (day 14 p.c., **lane 2**) and non-pregnant rats (**lane 3**) were reacted with anti-C-terminal peptide antiserum. A placental extract (60 μg of protein) served as control (**lane 1**). The arrows in b indicate the specifically detected antigens. The sizes of the molecular weight markers are indicated in the left margins.

DISCUSSION

In this paper, we have demonstrated by PCR and in situ hybridization that expression of the rat PSG gene *rnCGM1* is restricted to cells of the placenta, which are derived from the trophectoderm of the developing embryo. Recently, it was shown by in situ hybridization that human PSGs are synthesized in the syncytiotrophoblast, which corresponds to the rodent trophoblast giant cell layer (Lei et al., 1992). Negligible amounts of *rnCGM1* transcripts, or none whatsoever, could be detected in fetal liver or in salivary glands of adult rats, respectively. In humans, these tissues have been found to contain significant amounts of mRNA from various PSG genes, as estimated from the frequency of PSG-related cDNA clones identified in cDNA libraries (Zimmermann et al., 1989; Khan et al., 1989; Zoubir et al., 1990). This indicates either a general functional difference of PSGs in man and rat or, alternatively, a more specialized function of *rnCGM1*, which differs from other known rodent PSGs in having two additional IgV-like domains (Rudert et al., 1992; Chen et al., 1992).

The *rnCGM1* protein was identified by Western blot analyses in placental extracts as a 124 kDa antigen using three different antisera, two of which react with distinct epitopes in the N1 domain and the carboxyterminal region, respectively. This protein is secreted into the maternal blood like human PSG and the presumed murine PSG homologue PAMP-2 (Lin et al., 1974; Hau and Skovgaard Jensen, 1987). Its smaller size (116 kDa) in the blood may be caused by proteolytic cleavage or other modifications. Proteolytic truncation, however, probably does not occur at the carboxyterminal end of the molecule, since the anti-C-terminal peptide antiserum still reacts with the serum form. The observed size of *rnCGM1* proves that the four internal truncated leader regions (Rebstock et al., 1990) do not function as signal peptidase recognition sequences (von Heijne, 1986). From the primary amino acid sequence a relative molecular mass (M_r) minus leader of 74.6 kDa can be predicted for *rnCGM1* after removal of the signal peptide (Rebstock et al., 1990). The size difference between the native translation product and the mature protein is probably caused by extensive glycosylation which is also observed for the other members of the PSG/CEA family (reviewed in Thompson et al., 1991). Assuming an even distribution of the carbohydrate residues, a carbohydrate chain of M_r 3100 would be attached to each of the 16 potential N-glycosylation sites of *rnCGM1*. Using this same value, the rat and mouse PSGs containing three N domains and 6–8 putative N-glycosylation sites (Chen et al., 1992; Rudert et al., 1992) would have a M_r of 68–74 kDa. Therefore, it appears likely that the 75 kDa antigen detected by the anti-N1 domain antiserum (Fig. 5a, lane 3) represents PSGs with three N domains. This is in agreement with the findings by Ogilvie and colleagues (1989), that explant basal zone tissue from rat placenta synthesize two heterogenous proteins cross-reactive with anti-human PSG antisera: a major 120 kDa, which probably corresponds to *rnCGM1* and a minor 75 kDa species. PAMP-2, the PSG homologue of the mouse has been reported to have a M_r of 70 kDa, which would correspond to the smaller PSG species (Hau et al., 1980). The broader reactivity of our anti-N1 domain antiserum should be valuable in detecting other members of the rat PSG family, not consistently recognized by anti-human PSG antisera.

In situ hybridization with a *rnCGM1*-specific probe has revealed *rnCGM1* transcripts in the secondary giant cell subpopulation, which lines the maternal decidua in day 14 placenta, but not in the primary trophoblast giant cells. The latter cells are derived from the mural trophectoderm, whereas the secondary trophoblast giant cells are decendants of the ectoplacental cone (Gardner, 1983). Despite this difference in origin the division between primary and secondary trophoblast cells is partly arbitrary, since both ancestral cell types represent continuous structures and cells from near the line of junction could give rise to either type (Snell and Stevens, 1966). Therefore, *rnCGM1* might help to differentiate trophoblast giant cells, being a marker for a subpopulation of secondary trophoblast

giant cells. The amount of *rnCGM1* mRNA in the spongiotrophoblast increases from day 14 to day 18. In day 18 placenta it is the only tissue that contains *rnCGM1* transcripts. Ogilvie et al. (1989) found by immunohistochemical studies a similar spatiotemporal tissue distribution for rat placental proteins, which cross-react with anti-human PSG antisera. The protein was detectable from day-12 p.c. onward. In similar studies, MacPherson and co-workers (1985) identified PSG-related proteins with anti-human PSG antisera in the spongiotrophoblast of rat placentae between day 10 and day 20 of gestation. Since anti-human PSG antiserum preferentially reacts with a 124 kDa PSG-related protein (Fig. 5a) and recognizes the *rnCGM1* protein produced by transfectants (data not shown), it can be concluded from the immunohistological studies that the *rnCGM1* protein is also present in those cells where *rnCGM1* transcripts are found.

The spatiotemporal distribution of *rnCGM1* in placenta is compatible with two possible hypothetical functions of PSGs: They could play a role in the specific suppression of the maternal immune system and/or in the process of invasion of the uterine decidua by embryonic trophoblast cells during implantation. These effects could be primarily mediated by secondary trophoblast giant cells, which are in close contact with maternal tissue and are massively invading the decidua especially at early stages of development. The functional role of *rnCGM1* synthesized in the spongiotrophoblast is unclear. From there it is probably secreted into the maternal blood. Despite the appearance of *rnCGM1* protein in the maternal circulation (Fig. 5b), a regionally restricted rather than a systemic function of rodent PSG is suggested by the observation by Hau et al. (1985) that intrauterine application of anti-murine PSG antibodies resulted in the loss of fetuses in pregnant mice.

The lack of expression of classical class I MHC molecules on trophoblast cells has been implicated in the unresponsiveness of maternal cytotoxic, MHC-restricted T cells toward the semi-allogeneic embryo (reviewed in Hunt and Orr, 1992). On the other hand, the highly invasive trophoblast cells have to be controlled to avoid formation of metastases in the maternal organism (Strickland and Richards, 1992). Natural killer cell-related, large granular lymphocytes (LGL) of the decidua are thought to provide one element of this control (King and Loke, 1991). In order to balance the action of LGLs, PSGs could interfere with the recognition of fetal trophoblast cells by maternal natural killer cells. Since an amino acid sequence motif related to the tripeptide sequence ArgGlyAsp (RGD) is present in most PSGs (Khan et al., 1992; Rudert et al., 1992), this could occur by blocking adherence of these cell populations via integrin cell surface receptors, some of which recognize this sequence motif (Hynes, 1992). Indeed, integrins are known to be involved in cell/cell interactions like platelet aggregation and cell adhesion between leukocytes and endothelial cells during evasation (Springer, 1990). The mechanism of interaction may be similar to that found for soluble snake venom disintegrins, which competitively inhibit platelet aggregation by binding to the integrin GPIIb/IIIa on the surface of platelets via an RGD motif (Blobel and White, 1992). Furthermore, it is possible that PSG directly promote invasion by disrupting integrin/extracellular matrix protein interactions within the basal lamina of the decidual epithelium. Experiments designed to investigate these possible functions of PSGs especially in earlier developmental stages are underway.

EXPERIMENTAL PROCEDURES

RNA Isolation

Tissues were taken from BDHII rats (Druckrey, 1971) that were anesthetized and killed by cervical dislocation and immediately frozen in liquid nitrogen. For mating, male and female BDHII rats were kept together for 24 hr. This day was designated day 0 of gestation. Total RNA from fetal tissues was isolated by the method described by Fiddes and Goodman (1979), RNA from adult tissues by the acid phenol method (Chomczynski and Sacchi, 1987). Poly(A) RNA was isolated by one round of chromatography on oligo(dT)-cellulose (Sigma) according to Aviv and Leder (1972).

PCR Analyses

Reverse transcription with avian myeloblastosis virus reverse transcriptase (Promega) and PCR with *Taq* polymerase (Promega) were slightly modified after Sambrook et al. (1989), as described by Lucas et al. (1991). Briefly, in a total volume of 50 μl, 100 pmol of a random hexamer mixture (Pharmacia) was used to prime cDNA synthesis from 1 μg of total RNA. DNA fragments were amplified during 30 cycles (denaturation: 1 min, 93°C; extension: 3 min, 72°C) in the presence of pairs of specific oligonucleotides (50 pmol each) using optimal annealing temperatures (see below) for 15 sec. In order to avoid amplification products of the same size from contaminating genomic DNA the primers were chosen from different exons:β-actin-5′,5′-ACGGCTGCTTCCAGCTCCTC-3′,β-actin-3′,5′-AGCCATGCCAATCTCAT-CTTGT-3′(annealing temperature: 50°C); c-abl-5′, 5′-ATGGGGCAGCAGCCTGGAAA-3′, c-abl-3′,5′-GTGATGTAGTTGCTTGGGAC-3′(60°C);rnCGM1-5′,5′-TTATGAGCTATGCTGGC-3′,*rnCGM1*-3′, 5′-CTACGCCTTTGTACCAG-3′(50°C), yielding DNA fragments of 525 bp, 406 bp, and 845 bp, respectively (Ponte et al., 1983; Ng et al., 1985; Bernards et al., 1987; Fainstein et al., 1989; Rebstock et al., 1990). Before analysis by agarose gel electrophoresis, samples were treated with RNase A (20 μg/ml) at 37°C for 15 min. The identity of the *rnCGM1* PCR product was proven by hybridization (Sambrook et al., 1989) with an internal 5′-end-labeled oligonucleotide (S16: 5′-AGTACATGTGCAACTCC-3′) after alkaline transfer of the DNA fragments to a charged nylon membrane (Hybond N^+, Amersham) according to the manufacturer's protocol. The blot was washed in 6× SSPE (1× SSPE

is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.4], 1 mM EDTA), 0.1% SDS at 54°C, i.e., 3°C below the calculated melting temperature (Lathe, 1985).

For the isolation of *rnCGM1* cDNA clones with extended 3′-untranslated regions the anchored PCR approach was used. 150 ng of poly(A) RNA from day-20 placenta were reverse transcribed with an oligo(dT)- and *Eco*RI-, *Sal*I-, and *Hin*dIII-linker-containing oligonucleotide (5′-GGAATTCGTCGACAAGC$(T)_{14}$G/C/A-3′) as a primer. This primer can hybridize at the ends of all polyadenylated mRNAs because of the redundancy at its 3′-end (Leibrock et al. 1989). Amplification was performed in 30 cycles (denaturation: 2 min, 93°C; annealing: 15 sec, 50°C; extension: 3 min, 72°C) after addition of 5′-primer S19 (pos. 1622–1638) from the N5 domain or S17 (pos. 2694–2710) from the 3′-untranslated region, located ca. 170 nucleotides upstream of the first polyadenylation signal (Rebstock et al., 1990). The subcloned cDNA fragments were sequenced using the T7 Sequencing Kit (Pharmacia). Specific primer selection for PCR was aided by the program Primer (Lucas et al., 1991).

In Situ Hybridization

Anesthetized pregnant Sprague Dawley Fisher rats (Charles River) were killed by cervical dislocation. Placentae and embryos were fixed separately in 4% formaldehyde in phosphate-buffered saline (PBS) for 24–48 hr, transferred for 24–48 hr into 0.5 M sucrose in PBS at 4°C for another 24–48 hr depending on tissue size. In situ hybridization was performed essentially as described by Huang et al. (1990). Briefly, tissue sections of 8–10 μm were hybridized with [^{35}S]labeled sense or antisense RNA probes (specific activity: $2–5 \times 10^8$ dpm/μg) and washed under highly stringent conditions as described. However, SSC was replaced by SSPE in the washing solutions. Probes were transcribed with the TransProbeT Kit (Pharmacia) from BlueScript containing the 1,171 bp SstI/EcoRI *rnCGM1* cDNA fragment (Rebstock et al., 1990). The template was linearized 3′ or 5′ of the insertion site with restriction endonucleases creating blunt or 5′-protruding ends. After autoradiography for 1–2 weeks nuclei were counterstained with hemalaun.

Expression of the *rnCGM1* N1 Domain in Bacteria

The coding region for the amino-terminal immunoglobulin variable-like domain N1 was amplified from full-length *rnCGM1* cDNA using the following primers: N1-5′,5′-ACGTAGATCTCAAGTCTCCATTGAATCC-3′,N1-3′,5′-ACGTAGATCTGAAGTACACATGAAGGTGC-3′. Both oligonucleotides contain *Bgl*II-linkers to aid cloning into the expression vector pDS56/RBII-6×His, which supplies a start codon and six histidine residues fused to the carboxy-terminus of the protein to be expressed (LeGrice and Grüninger-Leitch, 1990). The N1 domain expression construct was transformed into *E. coli* M15 containing the $lacI^q$-producing plasmid pDM1.1 (Certa et al., 1986). A two liter culture of the recombinant bacteria was grown to a density of $A_{600} \approx 0.7$ and induced to produce the recombinant protein by addition of β-D-thiogalactopyranoside to a final concentration of 400 μg/ml for 3 hr. The bacterial pellet was resuspended in 16 ml 50 mM sodium phosphate, pH 7.8, 1 mM phenylmethanesulphonyl fluoride containing 1 mg/ml lysozyme and incubated for 20 min on ice. After addition of NaCl to a final concentration of 300 mM, the suspension was sonicated for 3 min at 0°C, then centrifuged for 45 min at 100,000*g*. The pellet, which contained most of the recombinant protein, was resuspended in 30 ml of buffer A (50 mM sodium acetate, pH 6.2, 6 M guanidinium hydrochloride, 200 mM NaCl, 10 mM 2-mercaptoethanol), sonicated for 1 min on ice and centrifuged for 1 hr at 100,000*g*. The supernatant was applied to a Ni^{2+}-nitrilotri-acetate column equilibrated with buffer A. After extensive washing with buffer A, bound proteins were eluted with a pH gradient of 6.2–5.0, yielding two peaks. The eluted fractions were dialyzed against 1 M TrisCl pH 8.2, 300 mM NaCl, 10% (v/v) glycerol. The resulting precipitates were collected by centrifugation (13,000*g*, 30 min), washed twice with dialysis buffer, once with PBS, and resuspended in PBS. The individual fractions were analyzed by SDS polyacrylamide gel electrophoresis, whereby the peak eluting at lower pH was found to contain the recombinant protein.

Preparation of Antisera

Antisera were raised in rabbits against the recombinant N1 domain or the carboxyterminal *rnCGM1* synthetic peptide NH_2-LysThrSerLeuProValSerLeuAspValllelleGlu-COOH comprising the last 13 carboxyterminal amino acids of *rnCGM1* coupled to keyhole limpet hemocyanin (Calbiochem) in complete Freund's adjuvant (Muller, 1988).

Preparation of Placental Extracts and Western Blot Analyses

Protein extracts from whole rat placentae were obtained as described by Ogilvie et al. (1989). Sera from pregnant (day 14 p.c.) and non-pregnant rats were collected from the tail vein, coaggulated for 1 hr at room temperature and 1 hr on ice, centrifuged for 10 min at 200*g* and stored at −20°C. For Western analyses, proteins were size fractionated by SDS polyacrylamide gel electrophoresis and transferred to Immobilon P nylon membranes (Millipore). The membranes were blocked with 1% gelatine or 3% dried milk powder in 20 mM Tris/Cl, pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween 20 (Sigma) and immunostained after incubation with alkaline phosphatase-coupled goat anti-rabbit IgG antibodies using p-nitrotetrazolium blue (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrates.

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